Ras Gene Mutations: A Rare Event in Nonmetastatic Primary Malignant Melanoma

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Ras gene mutations have been implicated in the pathogenesis of a variety of human tumors. Mutated _ras_ genes have been isolated from human melanoma cell lines, but subsequent studies indicated that _ras_ gene mutations may be a rare event in melanocytic lesions. Recently, a study reported a high frequency of _ras_ mutations correlated with increasing invasion level. To address this inconsistency in the published data, we analyzed 50 primary melanomas to correlate invasion level, tumor thickness, histologic typing, and body localization with point mutations around codons 12/13/61 of the three _ras_ genes. After microdissection of paraffin-embedded tumor tissue, _ras_ gene mutations were analyzed by direct sequencing of tumor DNA amplified by polymerase chain reaction. Only two melanomas exhibited _ras_ gene mutations, one sample containing a transition from A to G at position 2 of N-_ras_ codon 61 and the other exhibiting a transversion from C to A at position 1 and a transition from A to G at position 2 of N-_ras_ codon 61. Both tumors were classified as Clark level IV, with a tumor thickness of 2.5 and 1.2 mm, respectively. Both were typed as superficial spreading melanoma and localized to intermittently sun-exposed body sites. The low frequency of _ras_ mutations in malignant melanoma and the lack of _ras_ mutations in melanoma samples from constantly sun-exposed body sites argue against the hypothesis of _ras_ mutations as a marker of progression in malignant melanoma and the suggestion that _ras_ mutations occur predominantly in melanomas from constantly sun-exposed body sites. _Key words: polymerase chain reaction/DNA sequencing/paraffin-embedded tissue. J Invest Dermatol 104:868–871, 1995_

Ras proto-oncogene proteins belong to the _Ras_ superfamily of GTP-binding proteins involved in the regulation of different intracellular processes, including cellular proliferation and differentiation, intracellular vesicular trafficking, oxidase generation, and cytoskeletal control (reviewed in [1]). The _ras_ proto-oncogene family—H-_ras_, Ki-_ras_, and N-_ras_—encodes 21-kD proteins that are similar structurally, functionally, and immunologically [2]. Activation of p21 ras has been shown to be regulated by a large number of guanine nucleotide-releasing stimulatory or GTPase-activating inhibitory proteins. GTP hydrolysis, resulting in inactive p21 ras, may be impaired by single amino acid substitutions at positions 12, 13, or 61. _In vitro_ mutagenesis studies have demonstrated that these substitutions represent a common mechanism of _ras_ activation and subsequent oncogenic cellular transformation [3].

Activated _ras_ genes have been detected in a wide variety of human tumors [2] and may be involved in tumor progression of malignant melanoma, as indicated repeatedly by _in vitro_ mutagenesis studies [4–8]. Melanoma cell lines showed predominantly N-_ras_ mutations at codon 61 and, at a lower frequency, H-_ras_ mutations. Subsequent studies of primary tissue specimens revealed point mutations almost universally in the N-_ras_ gene; H-_ras_ and Ki-_ras_ mutations may also occur at low frequency [7,9–12]. However, the frequency of mutated N-_ras_ genes in cultured and noncultured malignant melanoma cells has been demonstrated to be low, ranging from 5% to 20% [7,9,11]. Thus, it has been concluded that activated _ras_ genes may play no fundamental role in malignant melanoma development [7,9]. These results, however, did not exclude the possibility that _ras_ gene alterations may be restricted to a subset of melanomas, as indicated by analysis of primary melanoma samples from sun-exposed body sites [11].

Recently, one study specifying tumor thickness and invasion levels and analyzing tumor localization on body sites reported a high frequency of _ras_ mutations correlated with increasing Clark stage, i.e., 19% in Clark level II, 53% in Clark level III, 39% in Clark level IV, and 50% in Clark level V tumors [13]. Another study specifying histologic type and tumor localization reported a low frequency (6%) of N-_ras_ mutations in primary melanomas [14]. To address this inconsistency in the published data, we analyzed a series of 50 primary cutaneous malignant melanomas for mutations around codons 12, 13, and 61 of the H-_ras_, Ki-_ras_, and N-_ras_ genes by direct sequencing of microdissected, polymerase chain reaction (PCR)-amplified tumor DNA and correlated these results with clinicopathologic data including Clark level, Breslow thickness, typing, and tumor localization on body sites.

MATERIALS AND METHODS

Tissue Specimens Fifty cutaneous malignant melanomas from 50 patients, fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax, were taken from the files of the Department of Dermatology, University of Essen. For all tissue samples, clinicopathologic data including Clark level, Breslow thickness, typing, and localization on body sites were

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Abbreviation: p21 ras, _ras_ protein product of 21 kD.

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available from the clinical records. Histologic diagnosis and classification of all specimens were reexamined independently by two of the authors. These data are summarized in **Table I**.

Microdissection and Preparation of Genomic DNA Microdissection was performed as described [15] to reduce nonlesional tissue material, which may contribute to wild-type DNA. One to five serial paraffin sections were cut from each specimen, depending on the total area of the section processed. Histologic examination before and after microdissection confirmed the analysis of representative areas of the respective lesion **(Fig 1)**. Only tissue sections with Clark level and Breslow thickness according to those obtained by routine histologic analysis were included in this study. Remaining tumor tissue was placed in a reaction tube and subjected to DNA preparation as described [15,16].

Direct Sequencing of PCR-Amplified DNA PCR of Ha-*ras* codon 12/13/61, Ki-*ras* codon 61, and N-*ras* codon 12/13/61 was performed with 10 μl of sample DNA as described previously [17,18]. Ki-*ras* codon 12/13 was amplified in a nested PCR procedure essentially as described [15]. PCR products were purified by separation on a 5% non-denaturing polyacrylamide gel, elution, and precipitation according to standard techniques [19]. Purified PCR products were sequenced according to the dideoxy chain termination method [20] with amplification primers used as sequencing primers.

Table I. Tissue Samples Analyzed

Parameter	n
Clark level	
I	7
II	10
III	13
IV	11
V	9
Total	50
Breslow thickness (mm)	
<0.75	13
0.75–1.5	17
>1.5	20
Total	50
Type[a]	
In situ	7
LMM	8
SSM	22
NM	13
Total	50

[a] LMM, lentigo maligna melanoma; SSM, superficial spreading melanoma; NM, nodular melanoma.

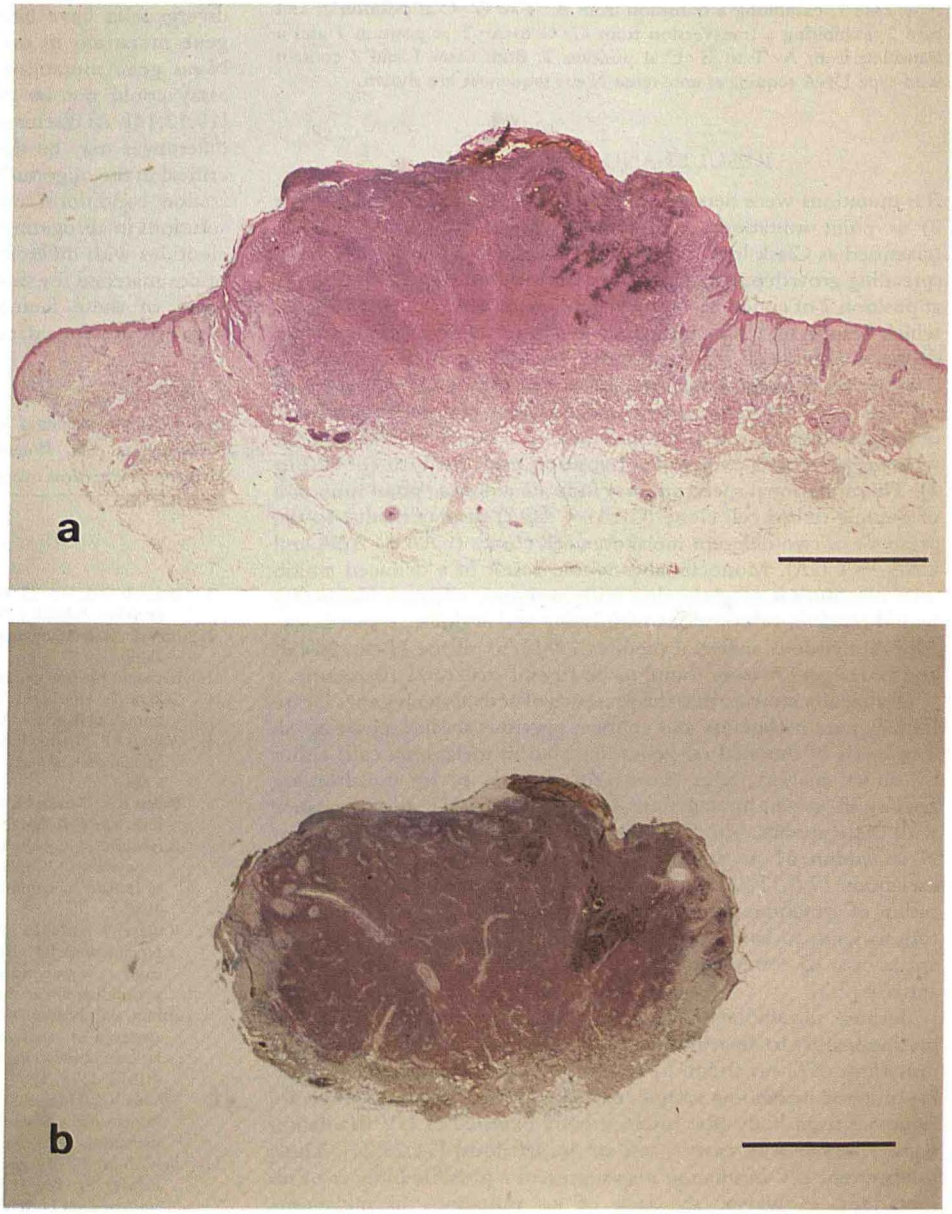

Figure 1. S100 immunostaining of microdissected tissue section reveals at least 80% tumor cells. Original tissue section before microdissection, with hematoxylin and eosin stain (*a*); and microdissected tissue section with S100 immunostaining and hemalaun counterstaining (*b*). *Bars*, 300 μm.

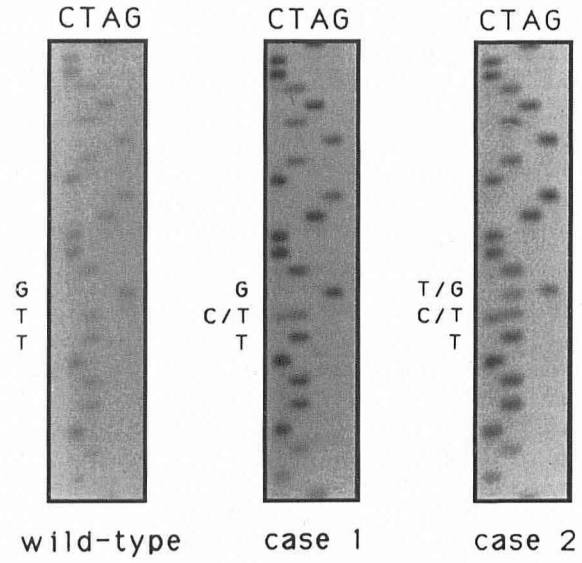

Figure 2. N-*ras* gene point mutations observed in two of 50 malignant melanoma tissues analyzed. Sequence analysis of N-*ras* 61 wild type; case 1, exhibiting a transition from A · T to G · C at position 2; and case 2, exhibiting a transversion from C · G to A · T at position 1 and a transition from A · T to G · C at position 2. Both cases 1 and 2 contain wild-type DNA sequence; anti-sense N-*ras* sequences are shown.

RESULTS AND DISCUSSION

Ras mutations were detected in two of 50 melanomas studied **(Fig 2)** as point mutations exclusively at N-*ras* codon 61. Case 1 (classified as Clark level IV, Breslow thickness 2.5 mm, superficial spreading growth pattern) exhibited a transition from A · T to G · C at position 2 of codon 61 of the N-*ras* gene (CAA → CGA) **(Fig 2)**, which results in a substitution of glutamine with arginine in the deduced amino acid sequence. Case 2 (classified as Clark level IV, Breslow thickness 1.2 mm, superficial spreading growth pattern) exhibited two different mutations in the same codon. At position 1 of N-*ras* codon 61, a transversion from C · G to A · T was present, whereas position 2 exhibited a transition from A · T to G · C **(Fig 2)**. This mutational spectrum may indicate a double point mutation of a single tumor cell clone (CAA → AGA) or may be due to the presence of two different melanoma cell clones (CAA → AAA and CAA → CGA). Monoclonality would result in a deduced amino acid substitution of glutamine with arginine, whereas biclonality would result in substitutions with lysine and arginine, respectively. All other codons analyzed (codons 12/13/61 of the H-*ras*, Ki-*ras*, and N-*ras* genes) were found to be free of structural alterations.

Our results indicate that the presence of activated *ras* genes is rare in malignant melanoma and confirm previous studies reporting on low levels of mutated *ras* genes detected in melanoma cells either by direct analysis, after short-term culture, or by transforming activity in *in vitro* mutagenesis studies [7,9,14,21]. In accordance with these studies, our results emphasize the first two positions of N-*ras* codon 61 as the site of *ras* gene mutations in malignant melanoma [7,9,11,21], with a resulting p21 ras mutation by substitution of glutamine with arginine or lysine [7,9,14]. These substitutions seem to have particular biologic significance because they are known to convert normal p21 ras into one with transforming activity [2,3].

Because ultraviolet (UV)-induced lesions seem to be targeted predominantly to dipyrimidine sites [22], it has been speculated that mutations of N-*ras* codon 61 result from UV irradiation [11]. Both *ras*-mutated melanoma samples exhibited N-*ras* mutations and were removed from body sites intermittently exposed to UV irradiation (case 1, upper arm; case 2, sole of the left foot) [11,23,24]. Thus, intermittent UV irradiation may represent a possible inducer of *ras* mutations. However, the lack of *ras* mutations in melanoma samples from constantly sun-exposed body sites as well as in the lentigo maligna melanoma subtype, which is associated with long-term constant UV exposure, argues against the suggestion that *ras* mutations occur predominantly in melanomas from constantly sun-exposed body sites [11].

In our study, the low frequency of *ras* gene mutations was not due to a low sensitivity level of our sequencing approach. Titration studies by mixing cloned wild-type and mutated *ras* gene sequences revealed a detection limit of 5% to 10% of mutated *ras* gene DNA for every amplification type and may result in the detection of at least 10% to 20% of mutated cells in tissue samples. This has been demonstrated previously for Ki-*ras* codon 12 [15]. The sensitivity of our approach was further enhanced by careful and histologically controlled microdissection of tumor material, with preservation of the deepest pathology of the tumor sample. Control immunostaining of randomly selected solitary tissue sections with S-100 antibody (Dianova, Hamburg, Germany) after microdissection revealed a portion of at least 80% tumor cells in the material subjected to DNA extraction **(Fig 1)**.

According to previous studies on primary tissue specimens of malignant melanoma, our study provides no evidence to support the hypothesis of *ras* mutations as a marker of progression in human malignant melanoma, as indicated by results of Ball *et al* [13] that were obtained by oligonucleotide hybridization assay. Similar divergencies have been reported previously for Ki-*ras* and N-*ras* gene mutations in melanocytic lesions; high levels of Ki-*ras* and N-*ras* gene mutations obtained by oligonucleotide hybridization assay could not be confirmed by direct sequencing approaches [10,12,14]. As discussed comprehensively by Albino *et al* [12], these differences may be due to a number of parameters known to be critical in the oligonucleotide hybridization assay, including hybridization conditions, use of tetramethylammonium-chloride–based solutions in abrogating melting-temperature differences of oligonucleotides with different purine content, and the use of oligonucleotides matched for similar structural features. It may be that one or more of these factors may be responsible for the differences between previous data and those of Ball *et al* [13].

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